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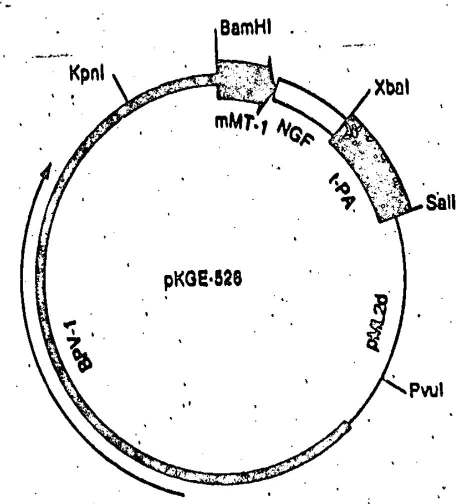
(54) Title: EXPRESSION OF HETEROLOGOUS PROTEINS USING EUCARYOTIC CELLS



(57) Abstract

A process for the production of a heterologous polypeptide or protein molecule through expression in a eucaryotic host cell, comprising the steps: (a) transforming said host cell by introducing therein an expression vector, said vector comprising an upstream regulatory sequence, a first DNA fragment encoding said molecule and positioned downstream of said regulatory sequence, which is capable of initiating RNA transcription of said DNA fragment, and further comprising a second DNA fragment with signals for efficient RNA processing located downstream of said first DNA fragment; (b) culturing the transformed host cell in a suitable medium under conditions allowing expression of the desired molecule; and (c) recovering the molecule resulting from step (b) from the culture; host cells transformed by introducing therein such an expression vector; and polypeptide and protein molecules prepared by such process.

EXPRESSION OF HETEROLOGOUS PROTEINS USING EUCARYOTIC CELLS

FIELD OF THE INVENTION

This invention relates to expression of gene products by recombinant DNA technology in a eucaryotic host.

BACKGROUND OF THE INVENTION

This invention relates to expression of gene products and production by recombinant DNA technology. More particularly the invention relates to expression and production employing a eucaryotic host.

Recombinant DNA technology has been used increasingly over the last years, for the expression and production of scientifically valuable and commercially important polypeptides. The focus has been on molecules of high pharmaceutical value, these include blood proteins as for example tissue type plasminogen activator (t-PA), coagulation factors VIII and IX, antithrombin III, erythropoietin as well as growth factors and hormones as for example growth hormon, insulin like growth factors, epidermal growth factor, tissue growth factors, nerve growth factors, and other important molecules as interferons, interleukins, monoclonal antibodies, receptors etc.

At present, even with the emergent recombinant DNA techniques, many of these proteins are usually purified from human blood or tissue, an expensive and time consuming process which carry the risk of transmitting infectious agents. Another limitation with this production is that the source for these molecules, may be restricted.

Although the expression of DNA sequences in bacteria to produce a desired polypeptide sometimes are attractive, it is a technology which has many restrictions.

In practice the bacteria often prove unsatisfactory as hosts because in the prokaryotic environment foreign polypeptides are unstable and not correctly processed. For example the carbohydrate structures attached to some mammalian polypeptides are not formed.

Recognizing these limitations, the expression of cloned genes in eucaryotic cells, especially in mammalian cells has in several cases proved to be a superior strategy.

The production of some molecules are very dependent on the combination of vector components and host cells. For example are mRNA processing signals, transcription initiation elements as well as a cell capable of effectively processing of the molecule such important parameters wh h have to be combined optimally in order to achieve an effective expression system.

The previous reported attempts to express high levels of human β-nerve growth factor has not been successful. Other molecules which are quite poorly expressed in recombinant DNA expression systems are human parathyroidea hormone and human coagulation factor VIII or derivatives thereof.

This invention provides a strategy for efficient production of these and other molecules in eucaryotic hosts, for therapeutic, diagnostic and related uses.

DESCRIPTION OF THE INVENTION

This invention provides a process for the production of heterologous polypeptides and protein molecules in eucaryotic cells. The system takes advantage of i: the high expression-vector copy number, ii: the efficient accumulation of the desired mRNA and iii: the broad host range of the system which allows the use of an optimal cell type.

In other aspects, the invention relates to cells which contain this expression system, to methods of producing polypeptides and proteins using these cells, and to the polypeptides and protein thus produced.

Accordingly, the present invention resides in a process for the production of a heterologous polypeptide or protein molecule through expression in a eucaryotic host cell. Said process comprises the following steps:

a) transforming said host cell by introducing therein an expression vect r, said vector comprising an upstream regula-

tory sequence, a first DNA fragment encoding said molecule and positioned downstream of said regulatory sequence, which is capable of initiating RNA transcription of said DNA fragment, and further comprising a second DNA fragment with signals for efficient RNA processing located downstream of said first DNA fragment;

b) culturing the transformed host cell in a suitable medium under conditions allowing expression of the desired molecule; and

c) recovering the molecule resulting from step b) from the culture.

The vector used in such process preferably also contains DNA sequences capable of stabilizing the mRNA of the molecule to be produced.

According to a preferred aspect of the invention the vector used contains as an upstream regulatory sequence the mouse metallothionein gene 1.

According to a further preferred aspect of the invention the vector used contains as a second DNA sequence a mammalian β-globin polyadenylation sequence.

The vector used may additionally contain a genetic element promoting an initial high and stable vector copy number.

The host cell used is preferably of mammalian origin including human cells, and cells of rodent origin are especially preferred.

Preferred embodiments of the process of the invention are directed to the production of NGF and t-PA, and for the production of human β-NGF the host cell is preferably of murine origin. For manufacturing t-PA it is preferred to use host cells of hamster origin.

The invention includes within its scope polypeptide or protein molecules prepared by the process outlined above, as well as host cells transformed by introducing therein an expression vector as described above.

VECTOR

The expression vector used for directing production of a heterologous protein consists of the following elements:

An upstream regulatory sequence, URS, capable to initiate RNA transcription of a downstream positioned DNA fragment.

This regulatory sequence has the RNA polymerase binding properties and may also bind to transactivating factors. The URS element also contains a sequence which acts as a starting position for the synthesized RNA molecule. The URS segment may also contain sequences which act as enhancer elements in some cells and regulatory elements which are inducible.

Known and analyzed URS elements are: metallothionein promoters and upstream regulatory sequences especially from mouse metallothionein gene 1 (mMT-1) or from human metallothionein gene 2a (hMT-IIA) as well as from other species of mammalian, insect or fish origin, heat shock promoters derived from different species, viral upstream regulatory elements for example simian virus 40 (SV 40) early and late promoters, cytomegalovirus early and immediate regulatory element from human or murine cytomegalovirus, different viral long terminal repeats (LTRs), different genomic fragments containing upstream regulatory elements.

The heterologous coding sequence of interest can be a cDNA or a genomic fragment encoding any heterologous peptide or protein. Coding sequences successfully expressed in the system are those encoding human β-nerve growth factor (β-NGF), human parathyroidea hormone (PTH), human plasminogen activator (t-PA), human blood clotting factor VIII (F VIII), somatomedin binding proteins (SMBP), growth hormones, lung surfactant apoproteins, plasminogen, antithrombin III, interferons, or derivatives of these molecules. Heterologous refers to a molecule introduced exogenously and/or a molecule produced by a host cell wherein the sequence encoding the molecule was intr duced exogenously. The molecules may be

identical with endogenously produced molecules or fragment thereof. Thus, the term heterologous refers to the origin of the molecule rather than the nature of the molecule.

A DNA fragment with signals for efficient RNA processing is introduced downstream of the coding element. This element may contain signals for transcription termination, polyadenylation and RNA stabilizing sequences. The vector construction may also include additional enhancers and/or RNA splice signals.

Examples of such elements analyzed which demonstrate efficient RNA processing are: β-globin polyadenylation sequences derived from mammalian species including human, preferably rodents such as rabbit or other species, different actin mRNA processing signals, viral RNA processing signals from SV 40, retroviruses, hepatitis virus etc.

In addition to the URS, the fragment encoding the desired molecule and the RNA processing signals, a genetic element that allows induced amplification, and amplifiable gene sequence (AGS), may also be included.

The AGS typically refers to a DNA encoding a protein which has a metabolic function such that in response to an environmental stimulus the level of protein production is increased, at least in part because of synthesis of multiple gene copies or amplification. The DNA element most frequently used for this purpose contains the murine metallothionein gene. This fragment allows amplification by the addition of heavy metals, i.e. cadmium or zink, to the culture medium. The metal concentration is increased stepwise, thereby selecting for the cell with the highest copy number and the highest expression. Other usable genetic elements that allow amplification are for example a dihydrofolate reductase encoding element and other fragments encoding some kind of drug resistance.

Also important in order to achieve high levels of expression is a genetic element that promotes an initial high and stable vector copy number, before a subsequent amplifica-

tion. This element may replicate efficiently in a large number of different cell lines derived from various tissues and species. This broad host range is of great value when special requirements on the cell line are of importance. An example of such genetic element are papilloma viruses, especially the Bovine papilloma virus.

In order to be able to grow the expression vector in bacteria a plasmid element is included. This plasmid element typically contains an origin of replication functional in bacterial systems and a genetic marker, for example a resistance gene.

HOST CELLS

The expression system of this invention is designed for production of peptides and proteins by recombinant DNA methods. Since many molecules of interest are human or mammalian proteins or peptides, host cells of eucaryotic origin especially mammalian cells are favoured. The use of eucaryotic host cells assures correct posttranslational modifications which are often of great importance for correcting the biological funtions of the proteins or peptides produced.

Host cells employed in combination with our expression system are eucaryotic cells, preferentially mammalian cells such as rodent cells. Cell lines frequently used are for example the murine C127 cell line (ATCC CRL 1616) and the hamster Don, BHK and CHO cell lines (ATCC CCL 16, ATCC CCL 10 and ATCC CCL 61, respectively).

Plasmid DNA, designated pKGE-526, pKGE-545 and pKGE-569 have been deposited in the collection of: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Maschoroder Weg 1b, D-3300 Braunschweig, Federal republic of Germany, on November 14, 1989 and have been identified there by accession numbers DSM 5637, DSM 5638 and DSM 5639, respectively.

Brief description of the annexed figures.

Fig. 1A. This figure describes the expression vector pKGE-526.

Fig. 1B. This figure describes the expression vector pKGE-545.

Fig. 1C. This figure describes the expression vector pKGE-569.

Fig. 2. This figure shows a Southern analysis of pKGE-545 in C127 cells. As a reference plasmid DNA purified directly from bacteria was digested with the following restriction endonucleases: XhoI which has no site in the vector, SacI which has a unique site in the vector (in the mMT-1 upstream regulatory sequence) and with Bam HI which has two sites in the vector (one at the fusion between BPV and mMT-1 elements and the other at the linker sequence between β-NGF cDNA and the rabbit β-globin element). The DNA from the transfected and stable transformed C127 cell line was digested with the same enzymes and also analysed undigested. The ^{32}P-labeled probe in this experiment was the human β-NGF cDNA, isolated as a ApaI and Hind III fragment, from pKGE-525.

Fig. 3. This figure shows an analysis of the relative β-NGF mRNA stability. mRNA was prepared from C127 cells which were producing β-NGF. The cells were cultured in the presence of the adenosine analogue 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazol (DRB), for different periods of time as indicated. mRNA was prepared and separated on agarose-formaldehyde gels, blotted to nitrocellulose filters and hybridized to ^{32}P-labeled human β-NGF and murine muscle actin probes, respectively. The results obtained indicate that the relative stability of the heterologous β-NGF transcript is lower in comparison to endogenous actin mRNA during the initial period of treatment. Measured during a long period the β-NGF mRNA seems to be more stable than the muscle actin transcript. Since the mRNA amounts are measured within the same sample, and the relative amounts are compared between the different samples, the error generated from different amounts to total RNA loaded on the gel can be neglected.

Fig. 4. This figure shows SDS-PAGE and western blot of conditioned medium from a pKGE-545 transfected C127 cell line, using a rabbit polyclonal anti-NGF antibody.

Fig. 5. Biological assay of NGF activity in conditioned medium from transfected C127 cells. Explanted sympathetic ganglia from chicken embryos are shown on dark-field micrographs after incubation with conditioned medium. (A) Fiber outgrowth when using medium from a transformed C127 cell line (clone 38) transfected with pKGE-526. The medium was conditioned for 24 h. (B) Lack of response when using medium from a tranformed C127 cell line transfected with pKGE-83. pKGE-83 is identical to pKGE-526 except that the cDNA encodes human t-PA instead of β-NGF.

EXAMPLES

The following examples are intended to illustrate but not to limit the invention.

Construction of the expression system of the invention, and the molecular biological characterization of it, employs standard methods generally known in the art of recombinant DNA field. For detailed description of the methods see Maniatis et al., 1982.

Cells were grown according to standard methods and according to instructions obtained from ATCC.

Example 1

Expression of human β-NGF

Expression vectors

The cDNA encoding the propro-polypeptide for human β-NGF (Nerve Growth Factor) was cloned as an Eco RI fragment into pUC 19, generating pKGE-525.

The complete amino acid sequence and nucleotide sequence as well as the natural processing of the propro-polypeptide generating the active mature 118 amino acid β-NGF molecule is described (Ullrich et al. 1983).

The cDNA fragment, approximately 850 bp, was isolated by restriction enzyme digestion with Ssp I and Apa I and agarose gel electrophoresis, fragment 1.

From another of our constructions, pKGE-32, the murine metallothionein-1 promoter and upstream regulatory signals were isolated as a Bam HI/ Hind III fragment of about 680 bp, fragment 2.

The expression vector pKGE-83 was cleaved with Apa I and Bam HI, a fragment containing the entire BPV genome, the pBR 322 derivative pML 2d and a human genomic fragment containing the t-PA transcription termination and polyadenylation signals were isolated as a 11.5 kb fragment, fragment 3.

Those three fragments described above were ligated and transformed in bacteria. This process generated the expression vector construct pKGE-526, fig. 1A.

In order to substitute the transcription terminator elements and mRNA processing signals in the β-NGF transcriptional unit pKGE-526 was digested with Bam HI and Apa I, and the fragment containing the murine MT-1 upstream regulatory element was isolated by low melting point agarose electrophoresis. This fragment was combined in a ligation reaction with: i, a pUC 18 Bam HI/Eco RI fragment containing the ampicillin resistance gene as well as replication signal and ii, a fragment from the construct pKGE-147, containing the 3' region of the translated part of human t-PA (tissue type plasminogen activator) cDNA and a cloning multilinker cloned close to the stop codon.

This ligation mixture was added to competent HB 101 _E. coli_ bacteria and several transformants were isolated and analysed. The resulting construct can be described as follows: The Bam HI/Eco RI pUC 18 plasmid element carrying the mMT-1 upstream regulatory element cloned in its 5' to Bam HI and the human β-nerve growth factor prepro-polypeptide encoding cDNA fused to a small part of the human t-PA 3' coding cDNA with a multi-linker cloning cassette fused to the Eco RI site. This construction is designated pKGE-532.

The pKGE-532 construct was then digested with Bam HI and Xba I, and the fragment containing mMT-1, the prepro-polypeptide coding fragment and a downstream t-PA fragment with suitable restriction enzyme sites, generating fragment A.

The entire bovine papilloma virus type 1 genomic fragment of approximately 7.9 kb and the approximately 2.3 kb large pML2d plasmid element was isolated as a common Bam HI/Sal I fragment of about 10.2 kb, designated fragment B.

The rabbit β-globin fragment of approximately 1.3 kb was isolated as Sal I/ Xba I fragment from the construct pKGE-195, generating fragment C. This fragment contains mRNA processing signals. The three fragments, A+B+C, were ligated and used to transform E. _coli_ bacteria strain HB 101. Transformants were isolated and analysed, some of them contained the expression vector pKGE-545 as a plasmid element (Fig. 1B).

In order to achieve higher expression levels of active human β-NGF the murine metallothionein gene was introduced into this vector. The introduction enables the amplification of expression vector copy number by stepwise increasing the concentration of some ions, cadmium or zink ions are most frequently employed, in the culture medium.

The same Bam HI/Xba I mMT-1 and β-NGF fragment (fragment A) as well as the same Sal I/ XbaI rabbit β-globin element (fragment C) used in the construction of pKGE-545 (described above) were used.

However, fragment B was substituted by another DNA sequence. The genomic fragment containing the upstream regulatory region and the coding exon sequences as well as the intron sequences was isolated linked to the entire bovine papilloma virus genome and the pML2d plasmid element as a Bam HI/ Sal I fragment of approximately 12 kb. This fragment is designated D.

These three fragments (A+C+D) were ligated and transformed int E. _coli_ bacteria by standard methods. Several

transformants were isolated and analysed and bacteria harboring the expression vector pKGE-569 as a plasmid element were identified, fig. 1C.

Transfection of host cells and selection of cell clones

The expression vectors described above were transfected into mouse C127 cells and hamster Don cells by use of the calcium phosphate method (Graham and Van der Eb 1973). The cells were cotransfected with the vector pKGE-53 which contains the gene coding for neomycin resistance under control of Harvey Sarcoma 5´LTR (long terminal repeat).

The neomycin analog G 418 was added to the media, and several clones were isolated after approximately 2-3 weeks of cultivation in G 418 containing medium.

The isolated clones were grown individually, and as a mixture representing the cell population, and assayed for expression of β-NGF.

β-NGF expression in the clones

Conditioned media samples from the various clones and the clone mixtures were initially assayed in a biological system. This assay is semi-quantitative and gives information about the biological characteristics of the molecule produced.

Nerve Growth Factor activity was assayed on sympathetic ganglia from 9-day-old chick embryos embedded in a gel of collagen (Ebendal 1989). Cultures were incubated at 37°C in a 5% CO_2 atmosphere and nerve fiber outgrowths from ganglia were examined in an inverted microscope. Density of fibres was compared against a standard of responses evoked by mouse β-NGF. Activities were expressed in biological units, BU.

The assay described above is then completed with an enzyme immunoassay (EIA), measuring the level of extracellular dimeric β-NGF antigen.

The EIA was developed originally for mouse β-NGF. The immunoassay components are commercially available from

Boehringer Mannheim, Bromma, Sweden. We have utilized the EIA for determination of human NGF essentially as described by Heinrich and Meyer (1988), except that microtiter wells were coated with monoclonal anti NGF antibody at a concentration of 50 ng/ml and the β-gal conjugated monoclonal anti NGF antibody was used at a dilution of 1:40.

The expression values obtained with the two different assay methods were in agreement with each other.

In comparison with data presented for β-NGF expression the expression values presented here are surprisingly high, and substantially higher than for all other expression systems.

Table 1

Cell line	C 127		Dan	
Expression				
($BU/10^6$ cells/day) (BU=Biological Unit)	mean	max	mean	max
Expression vectors				
pKGE-526	10	25	3	12
pKGE-545	80	100	N.D.	N.D.
pKGE-569	140	360	N.D.	N.D.

Example 2

Expression of human t-PA

Several nucleotides in 5'untranslated as well as most of the translated cDNA encoding human tissue type plasminogen activator (t-PA) (Pennica et al. 1983), was cloned as a Bam

HI and XmaI fragment of approximately 1.8 kb. This fragment was used to the genomic XmaI and EcoRI fragment, from the last exon and downstream of it, in the way that the remaining translated sequence was fused in frame thus generating the complete original translated sequence followed by the original t-PA mRNA processing signals. These two fragments were subcloned by ligation in pUC 18 at the Bam HI and Sal I sites.

In order to introduce this coding element in an expression vector the fragment was ligated with a DNA fragment of about 11 kb containing the entire BPV genome and the mouse metallothionein-1 (mMT-1) upstream regulatory element and the Bam HI/Sal I part of pML2d containing the ampicillin resistance encoding sequence and replication signals. The Bam Hi site at the 5' end of the t-PA sequence, was fused to the 3' end of mMT-1 at the Bgl II site located in the leader sequence and the 3' termini of the t-PA encoding fragment was fused at the Sal I site in pML2d. The transcription of BPV genes and the t-PA expression unit is unidirectional. This expression vector is designated pKGE-83.

For allowing induced vector copy amplification the genomic fragment containing the mMT-1 regulatory signals and all exons and introns was subcloned and the ends were converted to Sal I sites thereby facilitating isolation and introduction of the element as a Sal I fragment.

The pKGE-83 expression vector was digested with SalI and the linearized DNA vector was isolated and ligated with the SalI fragment containing the mMT-1 gene, described above. The ligation mixture was added to competent E. coli HB 101 bacteria and transformants were isolated and analysed. Bacteria containing the resulting expression vector with the mMT-1 gene fragment introduced with the same transcriptional direction as the t-PA expression unit were identified. Expression vector DNA was prepared and designated pKGE-183.

A third expression vector was constructed in which the mRNA processing signals, in pKGE-83, derived from human t-PA

was altered to such signals derived from the rabbit β-globin gene. The β-globin fragment was fused approximately 20 bp downstream of the t-PA stop codon, and contained piece of the second exon, the last intron and then the last exon and sequences located further downstream, the total length of the β βglobin fragment being about 2.8 kb. The resulting expression vector is designated pKGE-183.

These three vectors were transfected into C127 and Don cells, as described above (Example 1).

The extracellular t-PA antigen levels were determined using a commercial ELISA kit (BioPool, Sweden). Enzymatic activity was analysed with Coa-Set t-PA chromogenic assay (Kabi). The melanoma t-PA used as standard in both assays above was calibrated against the reference preparation of t-PA (83/517) obtained from the National Institute of Biological Standards and Control, London (U.K.).

Table 2

Cell line	C127		Don	
Expression (ng/10^6 cells/day)	mean	max	mean	max
Expression vectors				
pKGE-83	400	3080	1260	7600
pKGE-183 (-Cd)	420	N.D.	150	N.D.
(+Cd, 25uM)	3420	19040	6440	14050
pKGE-184	3390	11740	6890	33860

SUBSTITUTE SHEET

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Maniatis, T., Fritsch, E.F. and Sambrook, J.: Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennett, W.F., Yelverton, E., Seeburg, P.H., Heyneker, H.L., Goeddel, D.V. and Collen, D.: Cloning and expression of human tissue type plasminogen activator cDNA in E. coli. Nature 301 (1983) 214-221.

Ullrich, A., Gray, A., Berman, C. and Dull, T.J.: Human β-nerve growth factor gene sequence highly homologous to that of mouse. Nature 303 (1983) 821-825.

CLAIMS

1. A process for the production of a heterologous polypeptide or protein molecule through expression in a eucaryotic host cell, comprising the steps:

a) transforming said host cell by introducing therein an expression vector, said vector comprising an upstream regulatory sequence, a first DNA fragment encoding said molecule and positioned downstream of said regulatory sequence, which is capable of initiating RNA transcription of said DNA fragment, and further comprising a second DNA fragment with signals for efficient RNA processing located downstream of said first DNA fragment;

b) culturing the transformed host cell in a suitable medium under conditions allowing expression of the desired molecule; and

c) recovering the molecule resulting from step b) from the culture.

2. A process according to claim 1, wherein the vector used also contains DNA sequences capable of stabilizing the mRNA of the desired molecule.

3. A process according to claim 1 or 2, wherein the vector used contains as an upstream regulatory sequence the mouse metallothionein gene 1.

4. A process according to any preceding claim, wherein the vector used contains as a second DNA sequence a mammalian β-globin polyadenylation sequence.

5. A process according to any preceding claim, wherein the vector used additionally contains a genetic element promoting an initial high and stable vector copy number.

6. A process according to any preceding claim, wherein the host cell is of mammalian including human origin.

7. A process according to any preceding claim for the production of a molecule selected from: blood proteins, such as t-PA, coagulation factors VIII and IX, antithrombin III, erythropoietin, growth factors and hormones, such as growth

hormone, insulin like growth factors, epidermal growth factor, tissue growth factors, nerve growth factors, and interferons, interleukins, monoclonal antibodies, receptors, somatomedin binding proteins, and lung surfactant apoproteins.

8. A process according to claim 7 for the production of NGF or t-PA.

9. A process according to claim 8, wherein the host cell is of murine origin.

10. A process according to claim 9, wherein the host cell is of hamster origin.

11. A process according to claim 9 for the production of human β-NGF, wherein the host cell is of murine origin.

12. A process according to claim 9 for the production of t-PA, wherein the host cell is of hamster origin.

13. A polypeptide or protein molecule whenever prepared by the process of any preceding claim.

14. A host cell transformed by the introduction therein of an expression vector comprising an upstream regulatory sequence, a first DNA fragment encoding said molecule and positioned downstream of said regulatory sequence, which is capable of initiating RNA transcription of said DNA fragment, and further comprising a second DNA fragment with signals for efficient RNA processing located downstream of said first DNA fragment.

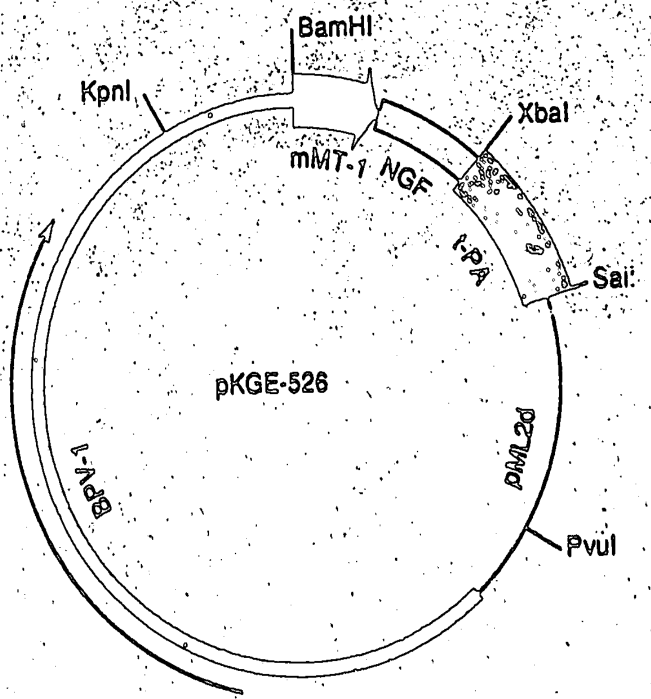


FIGURE 1A

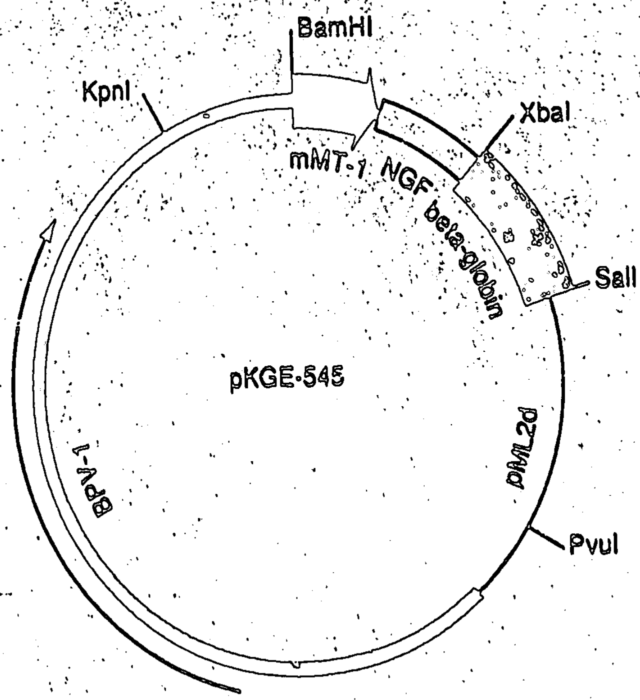

BamHI
KpnI
XbaI
mMT-1
NGF
beta-globin
SalI
pKGE-545
pML2d
BPV-1
PvuI

FIGURE 1B

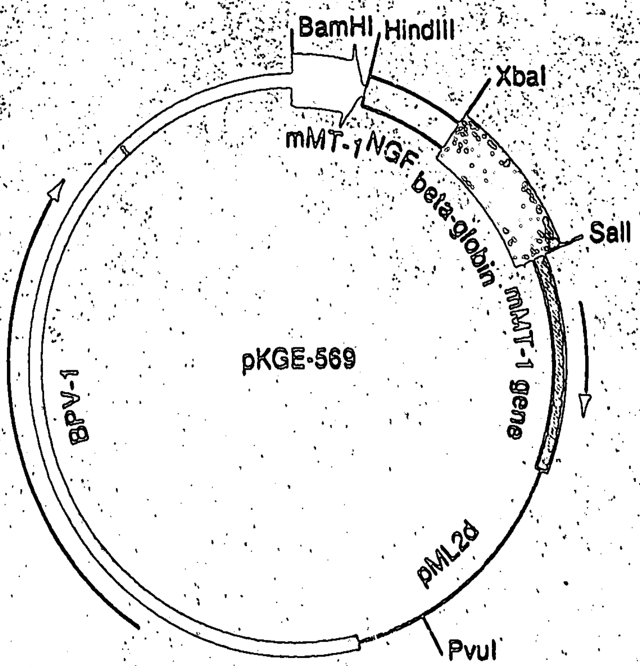

BamHI
HindIII
XbaI
SalI
mMT-1
NGF
beta-globin
mMT-1 gene
pKGE-569
BPV-1
pML2d
PvuI

FIGURE 1C

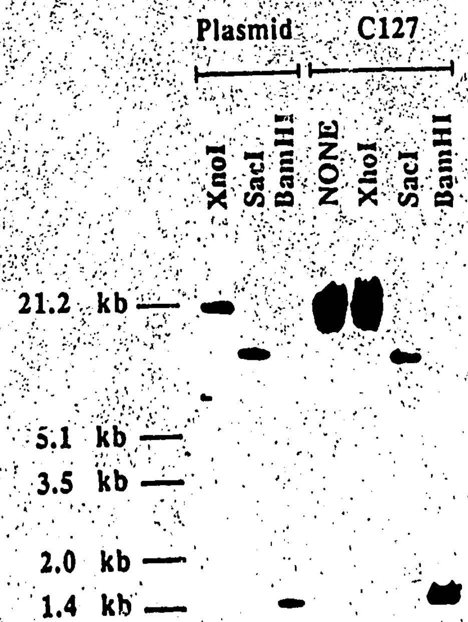

Plasmid
C127
XhoI
SacI
BamHI
NONE
XhoI
SacI
BamHI
21.2 kb
5.1 kb
3.5 kb
2.0 kb
1.4 kb

FIGURE 2

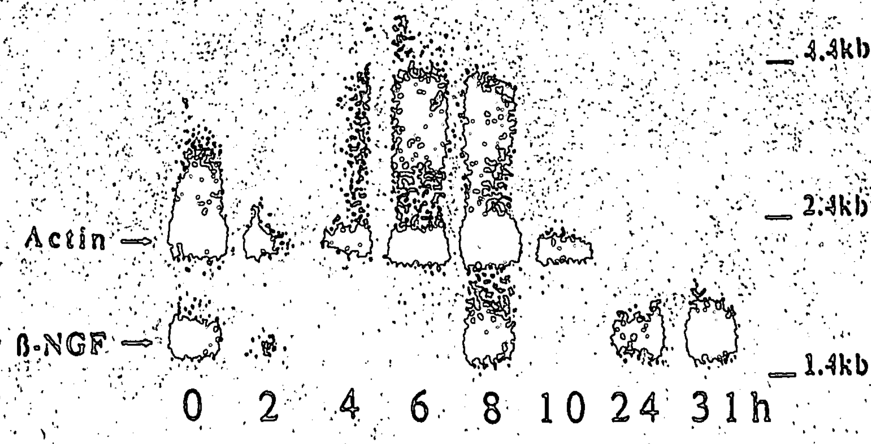
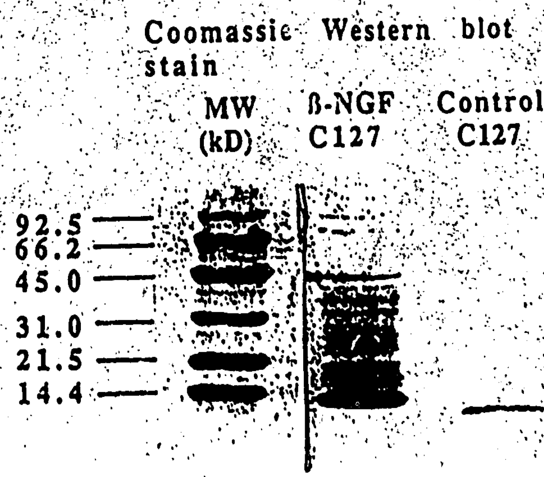


FIGURE 3



Coomassie stain
Western blot
MW (kD)
ß-NGF C127
Control C127
92.5
66.2
45.0
31.0
21.5
14.4

FIGURE 4

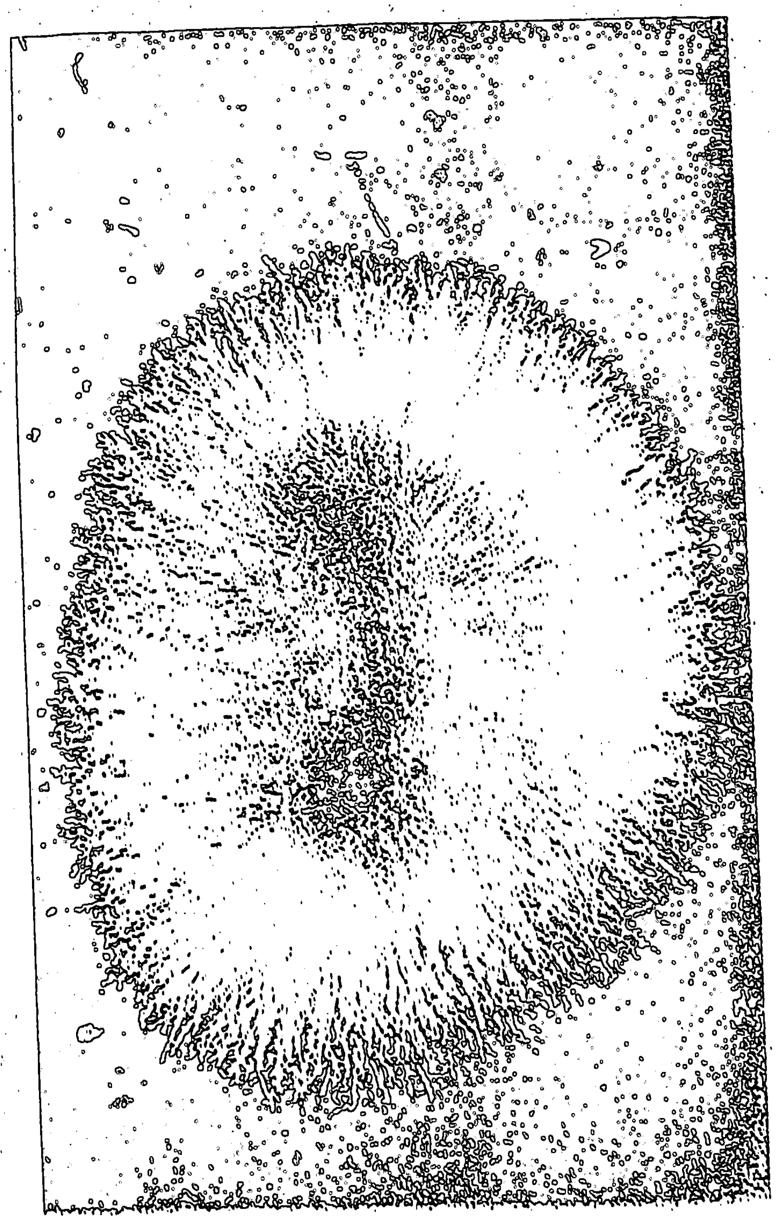


FIGURE 5A

FIGURE 5B